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Antimicrobial peptides, disease severity and exacerbations in Bronchiectasis

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Study design: OS, SA, JDC. Patient recruitment and data collection: OS, LP, AS, GSC, ART, FB, SA, JDC. Performed experiments and sample processing; LP, EC, AS, DC, AS, HRK, MO, SO, SV. Writing the manuscript: OS, LP, JDC. Revising of the manuscript and approval of submission: all authors. Responsible for the overall content as guarantors: OS, JDC.

Short title: Antimicrobial peptides in Bronchiectasis

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Key messages

What is the key question? Is there a dysregulation in pulmonary and systemic antimicrobial peptides (AMP) associated with the frequent exacerbator phenotype in bronchiectasis?

What is the bottom line? Frequent exacerbators have dysregulation of AMPs with particularly high levels of the cathelicidin LL-37 and low levels of secretory leukocyte protease inhibitor (SLPI) in sputum, which could predict time to next exacerbation and the frequency of exacerbations during follow-up.

Why read on? This is the first study to describe AMP dysregulation in the frequent exacerbator phenotype in bronchiectasis.

ABSTRACT

Rationale: Recently a frequent exacerbator phenotype has been described in bronchiectasis but the underlying biological mechanisms are unknown. Antimicrobial peptides (AMP) are important in host defense against microbes but can be proinflammatory in chronic lung disease.

Objectives: To determine pulmonary and systemic levels of AMP and their relationship with disease severity and future risk of exacerbations in bronchiectasis.

Methods: A total of 135 adults with bronchiectasis were prospectively enrolled at three European centers. Levels of cathelicidin LL-37, lactoferrin, lysozyme and Secretory Leukocyte Protease Inhibitor (SLPI) in serum and sputum were determined at baseline by ELISA. Patients were followed-up for 12 months. We examined the ability of sputum AMP to predict future exacerbation risk.

Measurements and main results: AMP levels were higher in sputum than in serum, suggesting local AMP release. Patients with more severe disease at baseline had dysregulation of airway AMP. Higher LL-37 and lower SLPI levels were associated with bronchiectasis severity index (BSI), lower FEV_1 and *Pseudomonas aeruginosa* infection. Low SLPI levels were also associated with the exacerbation frequency at baseline. During follow-up, higher LL-37 and lower SLPI levels were associated with a shorter time to the next exacerbation, while LL-37 alone predicted exacerbation frequency over the next 12 months.

Conclusions: Patients with bronchiectasis showed dysregulated sputum AMP levels, characterized by elevated LL-37 and reduced SLPI levels in the frequent exacerbator phenotype. Elevated LL-37 and reduced SLPI levels are associated with *Pseudomonas aeruginosa* infection and can predict future risk of exacerbations in bronchiectasis.

INTRODUCTION

Bronchiectasis is a chronic inflammatory lung disease characterized by permanent dilatation of the bronchi. Most patients suffer daily cough and sputum production and some of them experience frequent exacerbations (1). A frequent exacerbator phenotype has recently been described, in which patients consistently have multiple exacerbations over time and this condition independently predicts worse clinical outcomes including increased mortality (2). The underlying biological mechanisms leading to frequent exacerbations in this group have not yet been identified, but frequent exacerbators appear to have greater neutrophilic inflammation and are more susceptible to infection with bacteria, particularly *Pseudomonas aeruginosa* (3, 4).

Antimicrobial peptides (AMP) are important in host defense against pathogenic microbes in the lung (5). Among the most important and abundant antimicrobial peptides in the airway are lysozyme, lactoferrin and the cathelicidin LL-37 (which are proinflammatory mediators released from activated neutrophils, macrophages and bronchial epithelium) and Secretory Leukocyte Protease Inhibitor (SLPI; produced by respiratory epithelial cells and mostly anti-inflammatory) (6, 7). AMP function through multiple mechanisms including protein degradation (lysozyme), nutrient depletion (lactoferrin), cellular disruption and lysis, and inhibition of virulence factors (LL-37, SLPI) (8, 9). AMP are designed to protect against bacterial infection but in the context of chronic lung inflammation bacteria such as *P. aeruginosa* adapt to resist AMP killing, changing methods of iron acquisition and variations in biofilm-associated polysaccharides (10). The result may be an ineffective and excessive AMP response that stimulates inflammation without achieving bacterial clearance (11).

A consistent finding in bronchiectasis is the persistence of bacterial infection despite an excessive inflammatory response which instead of achieving bacterial clearance results in host damage (3, 12). The contribution of AMP to the vicious cycle of bronchiectasis has not been previously explored. We therefore hypothesized that AMP are altered in sputum from patients with bronchiectasis and may correlate with disease severity and predict future risk of exacerbations.

METHODS

Study design and Ethics

This was an international, multicenter, prospective, observational study which included consecutive adults with bronchiectasis. The study protocol was approved by local institutional review board and all subjects gave signed informed consent.

Participants

Patients were recruited from three regional specialist bronchiectasis clinics at the Hospital de la Santa Creu i Sant Pau (Barcelona, Spain), Ospedale Maggiore Policlinico (Milano, Italy) and Ninewells Hospital (Dundee, UK).

Bronchiectasis was defined by the presence of bronchial dilatation on high-resolution CT scanning with a compatible clinical history of cough, sputum production and/or recurrent respiratory infections. Patients with cystic fibrosis, primary immunodeficiency, active malignancy, active allergic bronchopulmonary aspergillosis, interstitial lung disease, active mycobacteria disease, HIV infection and long-term oral corticosteroid treatment were excluded.

Clinical assessments

All patients were clinically stable, defined by the absence of an exacerbation that required antibiotic treatment within 30 days. Frequent exacerbators were defined as 3 or more exacerbations per year at baseline. Demographic data, number of exacerbations in the previous year, relevant comorbid conditions and current treatments were recorded. Exacerbations were recorded as moderate (treated with antibiotics but not requiring hospitalization) or severe (requiring hospitalization or intravenous antibiotics). All patients underwent spirometry (13). The underlying etiology of bronchiectasis was determined after testing recommended by current guidelines (1, 14). The Bronchiectasis Severity Index (BSI) and FACED scores were calculated (15, 16). Severe disease was considered when BSI score was ≥ 9 points and FACED score was ≥ 5 points.

Longitudinal outcomes

Patients were followed-up for one year from the time of sputum and serum collection. During follow-up, patients were visited every 6 months as part of routine clinical practice at the study centers. Time to first exacerbation was calculated as the time from sampling to the first day of antibiotic administration because of an exacerbation.

Bacteriology

All bacteriology was performed on spontaneous early-morning sputum samples. Sputum was separated from saliva and the sample split for bacteriology and assessment of AMP levels. Samples were processed for bacteriology as previously described (17).

AMP and inflammatory mediator measurement

Methods of sputum and blood processing were standardized across the 3 sites. Sputum samples were processed by ultracentrifugation at 50,000g for 90 minutes followed by careful extraction of the supernatant. Samples were processed within 2 hours of expectoration and immediately frozen at -80C.

Serum and sputum Lactoferrin, Lysozyme (AssayPro, St. Charles, MO, USA), LL-37 (Hycult Biotech, Plymouth, PA, USA), SLPI and Matrix metallopeptidase 9 (MMP-9) (R&D Systems, Minneapolis, MN, USA) levels were measured by validated commercially available ELISA kits. Sputum samples were diluted 1/25000 for Lactoferrin and Lysozyme, 1/20 for LL-37 and 1/2000 for SLPI assays. The limits of detection were 0.625 ng/ml for lactoferrin, 0.0781 ng/ml for lysozyme, 0.14 ng/ml for LL-37 and 0.025 ng/mL for SLPI. Neutrophil elastase activity in sputum supernatants was measured by activity based immunoassay (ProAxsis LTD, Belfast, UK) as previously described (18).

Western Blot

To investigate the degradation of SLPI in bronchiectasis airway sputum the samples were subjected to denaturing gel electrophoresis on 15% SDS-PAGE gels before being transferred to nitrocellulose. After blocking with 5% skimmed milk in PBST the membranes were incubated overnight in anti-SLPI antibody at 0.1µg/ml (R&D cat no AF1274) at 4ºC. After incubation with Anti-goat HRP (BioRad, Watford (UK) cat no 1721034) at 1/2500 dilution for 1 hour the blot was washed thoroughly in PBST before SLPI proteins were visualized using Millipore Immobilon Western Chemiluminescent HRP substrate (cat no WBKLS0100) used as per manufacturers recommendation. A representative autoradiographic film is shown.

Air-liquid interface nasal epithelial cell culture

Nasal basal epithelial cells obtained from healthy and bronchiectasis patients were grown and seeded into 24-well transwell inserts at p2 or p3. Cells were fully differentiated into ciliated air-liquid interface cultures using Pneumocult ALI+ media (Stemcell technologies, Cambridge UK). Cells were apically treated with 100µl/well of PBS (control), Neutrophil Elastase at 3µg/ml (Sigma, Elastase from human leukocyte, E8140-1UN), with or without elastase inhibitor (AstraZeneca, AZD9668) at 10nM (a dose sufficient to completely block elastase activity as determined in preliminary experiments) or inhibitor alone. After 30mins of incubation at 37^{O}C, apical supernatants were collected. An additional 100µl/well PBS used to wash the apical surface of the cells and added to the corresponding supernatants. Baseline characteristics of healthy controls are described in the Methods section of the online data supplement.

Statistical analysis

Statistical analysis was performed using SPSS version 22. Categorical variables are presented as frequencies and percentages and comparisons performed using χ^2 or Fisher exact test when required. Continuous variables are presented as mean and standard deviation (SD) in parametrical variables and median and interquartile range (IQR) in non-parametrical ones. Differences were analysed using Student t-test, ANOVA test or their corresponding non-parametrical test when required (Kruskal-Wallis and Mann-Whitney tests). The relationship between linear variables was studied using linear regression and Spearman rank correlation (rho and p-value), with the latter included because relationships between inflammatory biomarkers were not assumed to be linear. Data are reported with the rho and p-values derived from the Spearman rank correlation. The contribution of different proteases to SLPI levels was studied with

multiple linear regression. Exacerbation rate was analyzed using a negative binomial model with time in study as an offset. Time to first exacerbation was modeled using Cox's proportional hazard regression. Exacerbation rate and time to first exacerbation were adjusted for BSI, site and fully adjusted for bronchiectasis severity, gender, aetiology, inhaled corticosteroid use and site. Patients lost to follow-up or dying within the 1 year follow-up were censored in the analysis. Discrimination between groups for prediction of hospitalization for severe exacerbations was tested using the area under the receiver operator characteristic curve. A p value <0.05 was considered significant.

RESULTS

Patient characteristics

A total of 135 adults with stable bronchiectasis were included in the study. **Table 1** shows the baseline characteristics of the population. Mean age was 69 (±10) years and 56% were females. The majority of patients had idiopathic or post-infective bronchiectasis and the mean BSI score was 8.0 (±4.3) and FACED score was 2.4 (±1.5), indicating moderate to severe bronchiectasis. Most of the patients (65%) were receiving inhaled long-acting bronchodilator treatment, whereas 43% were receiving inhaled corticosteroid treatment and 4% were receiving inhaled antibiotics. 322 exacerbations were recorded in the previous year, including 32 (10%) that require hospitalization. 68 patients (50.4%) were frequent exacerbators defined as 3 or more exacerbations per year at baseline. Frequent exacerbators had worse lung function and more severe disease compared to non-frequent exacerbators (see online table E1).

AMP levels in sputum and blood

All measured AMP in patients with bronchiectasis were higher in sputum than in serum. Lactoferrin was the AMP with highest pulmonary expression, with a median (IQR) of 114.6 (45.7-250.6) µg/ml. In serum, lactoferrin levels were 4.3 (2.3-6.5) µg/ml. Lysozyme was the AMP with highest systemic expression with a median (IQR) of 5.8 (4.4-7.3) µg/ml. In sputum, lysozyme levels were 68.9 (42.1-105.2) µg/ml. LL-37 levels were 1444 (28.1-8054) ng/ml in sputum and 328.3 (204.3-533.1) ng/ml in serum while SLPI levels were 536.4 (161.7-2729) ng/ml in sputum and 120.8 (85.6-152.2) ng/ml in serum.

No correlations between pulmonary and systemic AMP levels were found. Spearman rank correlation between sputum and serum lactoferrin was rho=-0.05 (p=0.59), between sputum and serum lysozyme was rho=0.06 (p=0.49), between sputum and serum LL-37 was rho=0.08 (p=0.36), and between sputum and serum SLPI was rho=0.03 (p=0.73).

AMP levels and disease severity

Patients with severe disease had elevated LL-37 levels and lower SLPI levels. Using the BSI score, the highest sputum LL-37 levels were detected in patients with severe BSI score in comparison to patients with moderate and mild disease (3835 (56.9-13502) vs 108.2 (21.1-6456) vs 148.7 (17.3-2743) ng/ml, p=0.007). Sputum SLPI levels were the lowest in the most severe group in comparison to moderate and mild patients (341.6 (88.8-1635) vs 608.5 (275.9-2678) vs 875.5 (444.1-7415) ng/ml, p=0.004) (**Figure 1**). Using the FACED score, similar relationships were observed to those seen with BSI although overall group differences were not statistically significant (p=0.05 and p=0.1 respectively, see online Figure E1). No correlation among systemic AMP levels and disease severity were found.

A weak inverse significant correlation was also observed between sputum LL-37 (rho=-0.25, p=0.004), lactoferrin (rho=-0.23, p=0.006) and lysozyme (rho=-0.19, p=0.02) with FEV_1, whereas sputum levels of SLPI had a weak correlation (rho=0.17, p=0.05) (see online Figure E2). No correlations between serum determinations and lung function tests were observed.

Frequent exacerbators had significant lower airway SLPI compared to infrequent and non-exacerbators (350.2 (101.7-2583) vs 560.7 (269.2-2424) vs 934.3 (385.1-7206) ng/ml, p=0.01). Sputum lysozyme, lactoferrin and LL-37 were not associated with baseline exacerbation frequency (see online Figure E3). Systemic AMP levels were not associated with exacerbation frequency.

AMP levels and airway infection

Sputum cultures were positive for bacteria in 86 patients (64%) and negative in 49 (36%). *P. aeruginosa* was the most frequently isolated pathogen in sputum culture (n=39, 29%), followed by *Haemophilus influenzae* (n=31, 23%), *Staphylococcus aureus* (n=6, 4%), *Escherichia coli* (n=3, 2%), *Moraxella catharralis* (n=2, 1%), *Stenotrophomona* (n=2, 1%), *Streptococcus pneumoniae* (n=2, 1%) and *Serratia marcensens* (n=1, 0.7%). Clinical characteristics of patients grouped according to the presence of *P.aeruginosa*, other pathogens or negative sputum culture (who were considered non-infected) are showed online (see online Table E2).

When compared to patients infected by other pathogens and non-infected patients, bronchiectasis patients with *P.aeruginosa* showed significantly higher sputum LL-37 levels (3835 (72.2-12435) vs 2425 (36.2-9658) vs 57.8 (17.1-3651) ng/ml, p=0.002), higher sputum lactoferrin levels (190.7 (68.1-364.3) vs 99.2 (40.4-250.6) vs 79.3 (45.7-201.1) ng/ml, p=0.04), and lower sputum SLPI levels (363.3 (103.7-739.7)

vs 753.3 (161.7-2624) vs 1066 (309.5-6245) ng/ml, p=0.001) (**Figure 2**). No differences in systemic AMP levels were found between patients infected by *P.aeruginosa*, patients infected by other pathogens and non-infected patients.

No differences in sputum AMP levels were found when patients with and without chronic macrolide treatment and inhaled corticosteroids were compared In addition, no differences were found among patients with and without nebulized antibiotics, although the small proportion of treated patients (n=6, 4%) did not exclude a potentially effect (see online table E3).

AMP and Elastase activity

A significant correlation between sputum LL-37 (rho=0.36, p=0.002), sputum lactoferrin (rho=0.38, p<0.001), sputum lysozyme (rho=0.26, p=0.03) and elastase activity were found (see online Figure E4). A previous study identified that in cystic fibrosis SLPI was degraded by neutrophil elastase into lower molecular weight cleavage products (19), while a further study found degradation by MMP-9 (20). We hypothesized that proteases activity in the bronchiectasis airway would be responsible for lower levels of SLPI. We found, however, no significant correlation between SLPI and elastase activity (rho=-0.09, p=0.46) or MMP-9 (rho=-0.13, p=0.2) (**Figure 3A and 3B**). In multiple linear regression incorporating elastase activity and MMP-9, proteases accounted for a maximum of 23% of the variance in SLPI levels.

By western blot, we identified a heterogeneous pattern of SLPI cleavage with no association between neutrophil elastase activity and SLPI cleavage pattern (**Figure 3C**).

Based on previous work that suggest neutrophil elastase can prevent release of SLPI from lung epithelial cells (21) we tested whether exogenous neutrophil elastase inhibited SLPI secretion from primary airway epithelial cells. We observed that nasal

epithelial cells from controls and bronchiectasis secreted similar amounts of SLPI (301.6 (167.4-676.4) vs 163.1 (128.8-340.4), p=0.46) (**Figure 3D**). The addition of exogenous elastase decreased the secretion of SLPI to 21.6 (13.8-37.7)% of the secretion from cells treated with control buffer (p=0.03). The NE inhibitor alone had no significant impact on SLPI release, while the addition of NE and inhibitor still resulted in inhibition of SLPI release suggesting that this effect was independent of NE protease activity (p=0.028) (**Figure 3E**).

Longitudinal outcomes

132 out of 135 patients (98%) completed 12 months of follow-up. During this time, a total of 310 exacerbations have been recorded in 102 patients, including 36 severe exacerbations. The median number of exacerbations per patient was 2.0. In univariate analysis, patients with 3 or more exacerbations during follow-up had higher levels of LL-37 (2976 (48.8-15472) vs 416.4 (21.8-7251) vs 61.4 (19.8-2865) ng/ml, p=0.02) and lower levels of SLPI (316.3 (89.9-944.1) vs 753.3 (343.1-3914) vs 1261 (265.2-6521) ng/ml, p<0.001) in comparison to those patients with 1 or 2 exacerbations and patients without exacerbations (**Figure 4**).

In an exploratory analysis we examined outcomes for patients above and below a cut-off representing the median of the population (to the nearest whole number). Patients with LL-37 ≥1500 ng/ml had a higher frequency of exacerbations (Incidence Rate Ratio (IRR) 1.57, 95%CI 1.04-2.37, p=0.02). Patients also had a shorter time to next exacerbation with LL-37 levels above the median for the population (Hazard Ratio (HR) 1.68, 95%CI 1.13-2.50, p=0.01). These findings persisted after adjusting for BSI and site (see table E4 in the online data supplement). LL-37 showed an area under the curve (AUC) of 0.76 (0.65-0.86), p<0.0001 for predicting hospitalization (see online

Figure E5).

For SLPI, low levels were not significantly associated with a higher frequency of exacerbations (IRR 0.76, 95%CI 0.50-1.51, p=0.1) but did demonstrate a shorter time to next exacerbation when a cut-off of 1000 ng/ml was used (HR 0.63, 95%CI 0.42-0.94, p=0.02) (**Figure 4**). After adjusting for BSI and site, a trend to predict the time to first exacerbation was observed (see online table E4). SLPI showed an AUC of 0.74 (0.62-0.86), p<0.0001 for predicting hospitalization for severe exacerbation (see online Figure E5).

DISCUSSION

This study demonstrates that airway AMP, especially high LL-37 and low SLPI sputum levels are associated with disease severity, disease activity and airway infection in patients with bronchiectasis. In addition, patients with higher levels of LL-37 and lower levels of SLPI had a shorter time to the next exacerbation and an increase frequency of exacerbations during follow-up. The relationships between LL-37/SLPI and both prior and future exacerbations suggest that dysregulation of AMP may be one of the mechanisms underlying the frequent exacerbator phenotype in bronchiectasis. Our findings suggested that AMP may be ineffective as an innate host defense mechanism, especially in those bronchiectasis patients with severe disease and with chronic *P. aeruginosa* infection. This fact may contribute to maintaining a vicious cycle of increased inflammation without eliminating bacterial infection. If further validated in prospective larger studies, LL-37 and SLPI could represent novel biomarkers to predict future risk of exacerbations in patients with bronchiectasis.

AMP are important in host defense against bacteria, viruses and fungi (5). They are produced by respiratory epithelial cells, neutrophils and macrophages and play an

important role in innate lung defense (6, 8). Although no previous comprehensive studies of AMP in bronchiectasis have been conducted, our results complement and extend previous observations in other chronic airway diseases such as Chronic Obstructive Pulmonary Disease (COPD) and Cystic Fibrosis (CF). In COPD, increased sputum levels of LL-37 were associated with airflow limitation, health status and exercise tolerance (22), and lower sputum lysozyme and SLPI levels were detected in patients with bacterial colonization and during acute infectious exacerbations (23). In bronchiectasis, one previous study demonstrated that high levels of sputum LL-37 were detected in patients chronically infected with *P. aeruginosa* (24). In our study, we demonstrated that elevated sputum LL-37 and reduced sputum SLPI levels were associated with disease severity, worse lung function and the presence of airway infection, especially due to *P. aeruginosa*. In addition, sputum lactoferrin and lysozyme levels were also associated to worse lung function and sputum SLPI were lower in frequent exacerbators. Local production of AMP in the lung, probably related to chronic airway inflammation, is likely to be the key mechanism in bronchiectasis, since we observed much higher levels in sputum than in serum and no relationship between serum levels and disease severity.

LL-37 is a human cathelicidin produced by neutrophils and epithelial cells in response to pro-inflammatory stimuli including cytokines, pathogen-associated molecular patterns and tissue injury (25). LL-37 displays antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi and viruses, neutralizes LPS activity, and protects against endotoxic shock (26). In addition, it is involved in the regulation of inflammation, cell proliferation and apoptosis (27). In our study, we demonstrated that high pulmonary levels of LL-37 are associated to disease severity, worse lung function and airway infection. It may seem contradictory that the presence of airway infection

and exacerbation risk is associated with higher levels of an AMP that should reduce infection. However, previous studies primarily in CF have demonstrated that the interaction of LL-37 with DNA and with glycosaminoglycan in the inflamed lung impairs the antibacterial function of LL-37 and its ability to neutralize LPS (28, 29). LL-37 is therefore able to enhance mucus production and promote inflammation while losing its antimicrobial activity (30). These previous findings likely explain the high levels of LL-37 identified in our study and how LL-37 may contribute to poor outcomes.

Bacterial clearance is the primary function of AMP. However, some pathogens, especially *P. aeruginosa*, have developed different process of adaptation to innate immune clearance mechanisms in the lung, such as creation of biofilms, resistance to inflammasome-mediated clearance and resistance to AMP (11). A recent study evaluating *P. aeruginosa* populations using whole-genome sequencing in patients with bronchiectasis showed that *P. aeruginosa* populations adapt by accumulating loss-of-functions mutations. This fact leads to changes in phenotypes including different modes of iron acquisition and variations in biofilm-associated polysaccharides (10). These findings may explain why, despite an appropriate host response increasing AMP secretion, *P. aeruginosa* persists in the lungs and the elevated AMP levels could contribute to perpetuate an excessive inflammatory response, impacting negatively on clinical outcomes.

In contrast to the LL-37 data, in our study sputum levels of SLPI were lower in the most severe patients and in those infected by *P. aeruginosa*. The main function of SLPI is to protect local tissue from the detrimental consequences of inflammation, as a result of its anti-protease activity and anti-inflammatory properties (7, 31). Previous studies have demonstrated decreased levels of SLPI in patients with CF infected with *P.*

aeruginosa due to neutrophil elastase degradation (19). In keeping with the emerging data that CF and bronchiectasis have highly diverging pathophysiology we found a different relationship in bronchiectasis. SLPI levels were not related to sputum neutrophil elastase activity or MMP-9 levels. SLPI degradation was heterogeneous with multiple cleavage products and was unrelated to elastase activity in sputum. Most patients, even those with high levels of SLPI by ELISA, had evidence of SLPI degradation suggesting that functional SLPI deficiency may be present even in patients with preserved protein levels. Our data suggest that multiple proteases such as matrix metalloproteinases and cathepsins are likely contributing to SLPI deficiency in the airway. However, protease levels only accounted for a maximum of 23% of the variance in sputum SLPI levels, suggesting an important contribution from reduced SLPI production. In addition, our data demonstrates that elastase reduces release of SLPI from epithelial cells independent of its protease activity, as previous data suggested (21). Other studies have generated a variant of SLPI resistant to degradation by neutrophil elastase (32). However, our data has important therapeutic implications since neutrophil elastase inhibitors that are in development for the treatment of bronchiectasis would not be expected to restore normal SLPI function in view of our findings.

Our study has limitations. First, this study was exploring multiple biomarkers and so we acknowledge the risk of spurious associations as a result of multiple statistical comparisons. We addressed this, however, by looking at baseline data with multiple markers and then focusing our prospective follow-up study on LL-37 and SLPI only, markers which had shown a clear association with disease severity at baseline. The finding of consistent results during follow-up makes spurious associations very unlikely. Our results were consistent, biologically plausible and robust across all studied AMP.. Second, the absence of a control group prevents the comparison of AMP levels

of bronchiectasis patients and healthy subjects. And finally, although our study is the largest of AMP in bronchiectasis, using well characterized patients in multicenter and international study, it is not possible to clarify if dysregulated AMP levels are markers of disease severity more than a cause of exacerbation, and further studies are needed to better clarify this point. Our study did not explore how sputum biomarkers could be implemented into clinical practice, which may be limited because of the need for sputum processing.

In conclusion, we found that patients with bronchiectasis show dysregulated AMP characterized by elevated pro-inflammatory LL-37, lactoferrin and lysozyme with reduced anti-inflammatory SLPI. The frequent exacerbator phenotype is associated with increased levels of LL-37 and degradation of SLPI, providing the first biological characterization of this key phenotype.

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Table 1. Patient demographics, clinical characteristics and prior treatments.

	Patients (n = 135)
Age (mean ± SD)	69.1 (10.5)
Male	59 (43.7)
Smoking status	
Never	82 (60.7)
Ex-smoker	23 (17.0)
Current	30 (22.2)
Comorbidities	
Cardiovascular disease	32 (23.7)
Diabetes mellitus	13 (9.6)
Stroke	12 (8.9)
Treatment	
Inhaled bronchodilators	88 (65.2)
Inhaled corticosteroids	59 (43.7)
Inhaled antibiotics	6 (4.4)
Chronic macrolide therapy	31 (23.0)
FEV_1 (% pred) (mean ± SD)	78.3 (28.3)
FVC (% pred) (mean ± SD)	94.3 (27.0)
BMI (kg/m^2) (mean ± SD)	25.9 (5.6)
Aetiology	
Idiopathic	61 (45.2)
Post-infective	22 (16.3)
Post-tuberculosis	12 (8.9)
COPD	12 (8.9)
Rheumatoid arthritis	6 (4.4)
CTD	4 (3.0)
ABPA	4 (3.0)
Primary Ciliary dyskinesia	3 (2.2)
Inflammatory bowel disease	3 (2.2)
Asthma	3 (2.2)
Haematological malignancy associated immunodeficiency	2 (1.5)
Young syndrome	2 (1.5)
Specific antibody deficiency	1 (0.7)
Exacerbations previous 12 months	
0	24 (17.8)
1	28 (20.7)
2	15 (11.1)
3	20 (14.8)
4 or more	48 (35.6)
BSI (mean ± SD)	8.0 (4.3)
FACED (mean ± SD)	2.4 (1.5)

Data is expressed by number (%) as otherwise is indicated. FEV1: Forced Expiratory Volume in 1 second; FVC: Forced Vital Capacity; BMI: Body Mass Index; COPD: Chronic Obstructive Pulmonary Disease. CTD: Connective Tissue Diseases; ABPA: allergic bronchopulmonary aspergillosis BSI: Bronchiectasis Severity Index

FIGURE LEGENDS

Figure 1.-Association of sputum AMP levels and severity of disease. A: Sputum LL-37 levels and BSI score divided in mild (0-4 points), moderate (4-8 points) and severe (9 or more points), B: Sputum SLPI levels and BSI score divided in mild, moderate and severe. *p* values are obtained by Kruskal-Wallis test. Graphs are represented as mean and SEM.

Figure 2.- Sputum AMP levels (A: LL-37, B: SLPI, C: lactoferrin, and D: lysozyme) and the presence of airway infection. Patients are divided in non-infected, infected by other pathogens different than *P. aeruginosa* and infected by *P. aeruginosa*. *p* values are obtained by Kruskal-Wallis test. Graphs are represented as mean and SEM

Figure 3.-A: Relationship between sputum SLPI and neutrophil elastase activity. B: Relationship between sputum SLPI and sputum MMP-9 levels. Rho and p-value were obtained from Spearman rank correlation, while the dotted line is derived from linear regression. C: Western Blot demonstrating degradation pattern of SLPI in sputum. Each column represents an individual patient ordered from left to right in terms of increasing sputum neutrophil elastase activity (measured by immunoassay as described in the methods). Abbreviations (NE: Neutrophil Elastase, FL SLPI= full length secretory leukocyte protease inhibitor, CP1= cleavage product 1, CP2= cleavage product 2, CP3= cleavage product 3). D: SLPI secretion from nasal epithelial cells of control and bronchiectasis patients. E: SLPI secretion from control (CON1 and CON2) and bronchiectasis (BE) nasal epithelial cells untreated (control buffer) or treated with Elastase, Inhibitor or a mixture of Elastase and inhibitor.

Figure 4.- A: Sputum SLPI levels and Frequency of exacerbations during 1 year follow up. B: Time to next exacerbation. Percentage of patients free of exacerbation using LL-37 1500 ng/ml as a cut-off (Hazard Ratio (HR) 1.68 (95%CI 1.13-2.50, p=0.01). C: Sputum SLPI levels and Frequency of exacerbations during 1 year follow up. D: Time to next exacerbation. Percentage of patients free of exacerbation using SLPI 1000 ng/ml as a cut-off (HR 0.63 95%CI 0.42-0.94, p=0.02). *p* values in panel A and C are obtained by Kruskal-Wallis test. Graphs are represented as mean and SEM.

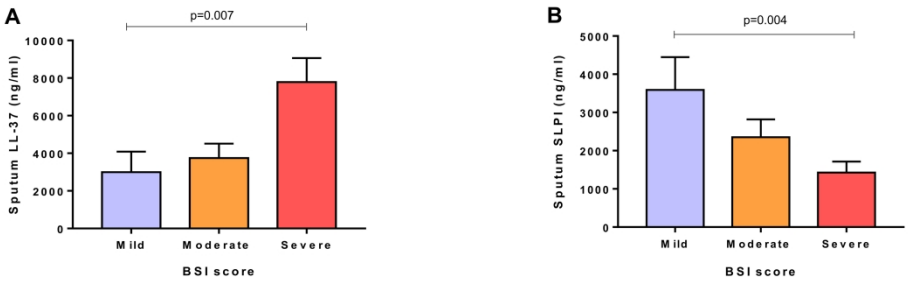

Figure 1.-Association of sputum AMP levels and severity of disease. A: Sputum LL-37 levels and BSI score divided in mild (0-4 points), moderate (4-8 points) and severe (9 or more points), B: Sputum SLPI levels and BSI score divided in mild, moderate and severe. p values are obtained by Kruskal-Wallis test. Graphs are represented as mean and SEM.

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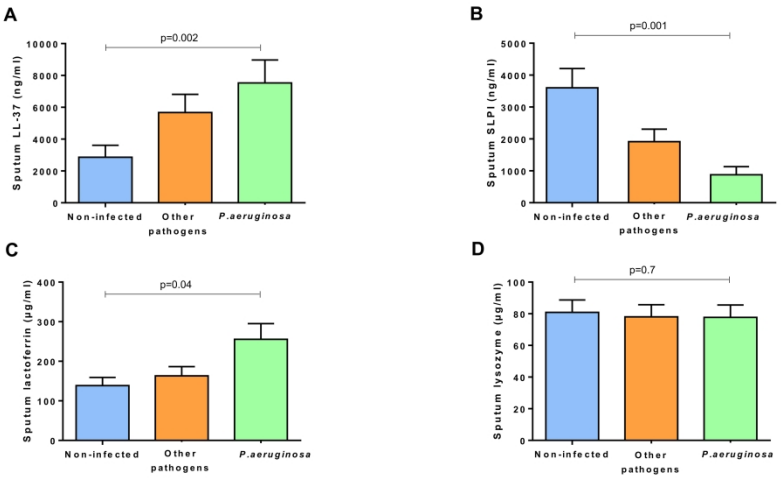

Figure 2.- Sputum AMP levels (A: LL-37, B: SLPI, C: lactoferrin, and D: lysozyme) and the presence of airway infection. Patients are divided in non-infected, infected by other pathogens different than P. aeruginosa and infected by P. aeruginosa. p values are obtained by Kruskal-Wallis test. Graphs are represented as mean and SEM

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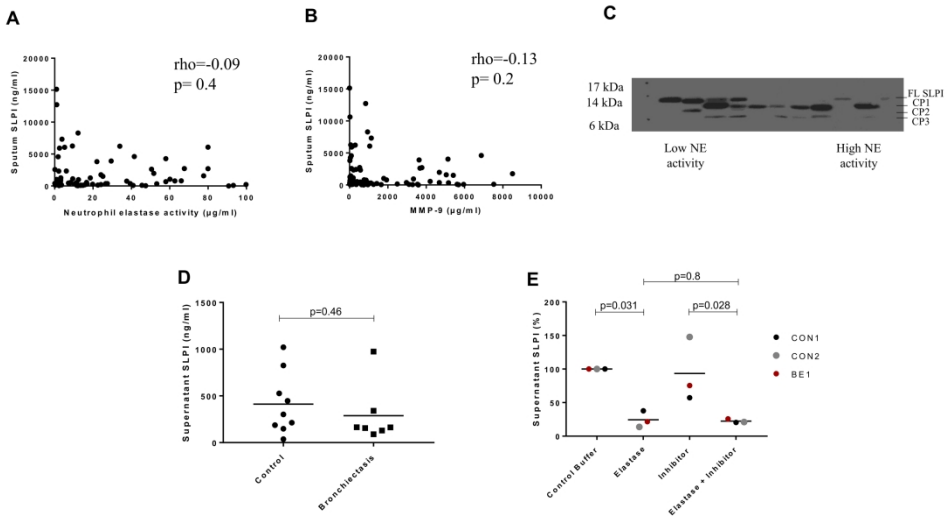

Figure 3.-A: Relationship between sputum SLPI and neutrophil elastase activity. B: Relationship between sputum SLPI and sputum MMP-9 levels. Rho and p-value were obtained from Spearman rank correlation, while the dotted line is derived from linear regression. C: Western Blot demonstrating degradation pattern of SLPI in sputum. Each column represents an individual patient ordered from left to right in terms of increasing sputum neutrophil elastase activity (measured by immunoassay as described in the methods). Abbreviations (NE: Neutrophil Elastase, FL SLPI= full length secretory leukocyte protease inhibitor, CP1= cleavage product 1, CP2= cleavage product 2, CP3= cleavage product 3). D: SLPI secretion from nasal epithelial cells of control and bronchiectasis patients. E: SLPI secretion from control (CON1 and CON2) and bronchiectasis (BE) nasal epithelial cells untreated (control buffer) or treated with Elastase, Inhibitor or a mixture of Elastase and inhibitor.

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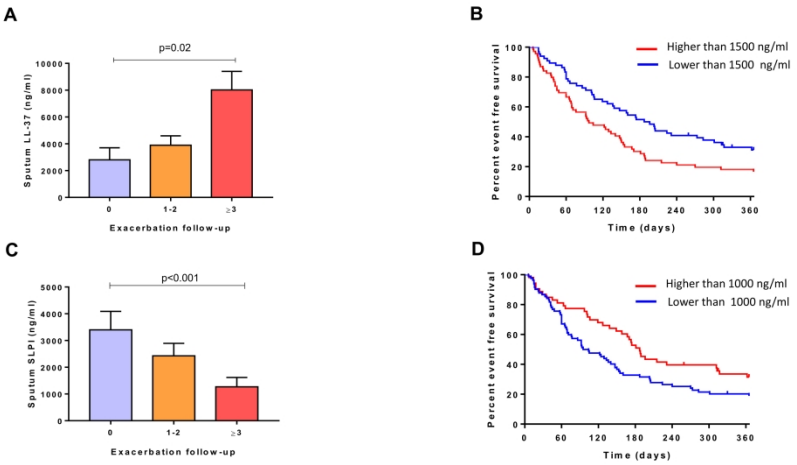

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Antimicrobial peptides, disease severity and exacerbations in Bronchiectasis

Oriol Sibila, Lídia Perea, Elisabet Cantó, Amelia Shoemark, Diane Cassidy, Alexandra Smith, Guillermo Suarez-Cuartin, Ana Rodrigo-Troyano, Holly R Keir, Martina Oriano, Samantha Ong, Silvia Vidal, Francesco Blasi, Stefano Aliberti, James D Chalmers.

ONLINE DATA SUPPLEMENT

METHODS

Air-liquid interface nasal epithelial cell culture

For the nasal cilia stuff, healthy controls were recruited from NHS Tayside and University of Dundee staff aged 25 to 60 years old. All controls had no history of rhinitis, asthma or other respiratory disease and were required to be free of upper respiratory tract infection for at least 6 weeks prior to nasal sampling.

RESULTS

AMP levels and FACED score

High sputum LL-37 levels were detected in patient with severe FACED score (7633 (719.1-21135) vs 2678 (30.1-9056) vs 329.6 (21.8-6412) ng/ml, p=0.05) while sputum SLPI levels were the lowest in the most severe group (236.2 (84.7-901.5) vs 769.7 (125.7-3592) vs 637.4 (296.0-3812) ng/ml, p=0.1) (**Figure E1).**

Table E1. Patient demographics, clinical characteristics and prior treatments among non-frequent exacerbators and frequent exacerbators.

	Non-frequent exacerbators (*n* = 67)	Frequent exacerbators (*n* = 68)	*P* value
Age (mean ± SD)	69.3 (±10.6)	68.7 (±10.5)	0.3
Male	32 (54.2)	27 (45.8)	0.3
Smoking status			
Never	44 (65.7)	38 (55.9)	0.1
Ex-smoker	13 (19.4)	10 (14.7)	
Current	10 (14.9)	20 (29.4)	
Comorbidities			
Cardiovascular disease	14 (20.9)	18 (26.5)	0.4
Diabetes mellitus	6 (9.0)	7 (10.3)	0.8
Stroke	5 (7.5)	7 (10.3)	0.6
Treatment			
Inhaled long-acting bronchodilator	35 (52.2)	53 (77.9)	0.002
Inhaled corticosteroids	24 (35.8)	35 (59.3)	0.07
Inhaled antibiotics	2 (4.0)	4 (7.0)	0.5
Chronic macrolide therapy	17 (25.4)	14 (20.6)	0.5
FEV_1 (% pred) (mean ± SD)	87.5 (±27.7)	69.3 (±26.2)	<0.0001
FVC (% pred) (mean ± SD)	101.6 (±26.1)	87.1 (±26.2)	0.004
BMI (kg/m^2) (mean ± SD)	26.4 (±4.4)	25.4 (±6.5)	0.06
Aetiology			
Idiopathic	33 (49.3)	28 (41.2)	0.1
Post-infective	12 (17.9)	10 (14.7)	
Post-TBC	8 (11.9)	4 (5.9)	
Others	14 (20.9)	26 (38.2)	
Exacerbations previous 12 months			
0	24 (35.8)	0 (0.0)	<0.0001
1	28 (41.8)	0 (0.0)	
2	15 (22.4)	0 (0.0)	
3	0 (0.0)	20 (29.4)	
4 or more	0 (0.0)	48 (70.6)	
BSI (mean ± SD)	5.8 (±3.0)	10.3 (±4.3)	<0.0001
FACED (mean ± SD)	2.1 (±1.4)	2.6 (±1.7)	0.1

Data are presented as n(%) unless otherwise indicated.

BMI, body mass index; BSI, Bronchiectasis Severity Index; FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; PPB, potentially pathogenic bacteria; TBC, tuberculosis; SD, standard deviation.

Table E2. Patient demographics, clinical characteristics and prior treatments among non-infected, infected by *P. aeruginosa* and infected by other pathogens

	Non-infected (*n* = 49)	Infected by other pathogens (*n* = 47)	Infected by *P. aeruginosa* (*n* = 39)	*P* value
Age (mean ± SD)	67.8 (10.6)	70.9 (10.6)	68.7 (10.3)	0.4
Male	17 (34.7)	24 (51.1)	18 (46.2)	0.3
Smoking status				
Never	31 (63.3)	24 (51.1)	27 (69.2)	0.2
Ex-smoker	9 (18.4)	7 (14.9)	7 (17.9)	
Current	9 (18.4)	16 (34.0)	5 (12.8)	
Comorbidities				
Cardiovascular disease	10 (20.4)	11 (23.4)	11 (28.2)	0.7
Diabetes mellitus	6 (12.2)	6 (12.8)	1 (2.6)	0.2
Stroke	4 (8.2)	4 (8.5)	4 (10.3)	0.9
Treatment				
Long-acting bronchodilator	28 (57.1)	27 (57.4)	34 (85)	0.01
Inhaled corticosteroids	15 (30.6)	24 (51.1)	20 (51.3)	0.07
Inhaled antibiotics	0 (0)	4 (8.5)	2 (5)	0.1
Chronic macrolide therapy	12 (24.5)	9 (19.1)	10 (25.6)	0.7
FEV_1 (% pred) (mean ± SD)	85.5 (23.5)	83.6 (29.3)	62.8 (27.3)	<0.001
FVC (% pred) (mean ± SD)	98.1 (23.7)	99.7 (26.2)	83.1 (29.2)	0.008
BMI (kg/m^2) (mean ± SD)	26.3 (5.6)	27.6 (5.7)	23.3 (4.2)	0.001
Bronchiectasis etiology				
Idiopathic	16 (32.7)	29 (61.7)	16 (41.0)	0.1
Post-infective	9 (18.4)	6 (12.8)	7 (17.9)	
Post-tuberculosis	6 (12.2)	1 (2.1)	5 (12.8)	
Others	18 (36.7)	11 (23.4)	11 (28.2)	
Exacerbations previous 12 months				
0	13 (26.5)	9 (19.1)	2 (5.1)	0.1
1	13 (26.5)	8 (17.0)	7 (17.9)	
2	5 (10.2)	4 (8.5)	6 (15.4)	
3	8 (16.3)	6 (12.8)	6 (15.4)	
4 or more	10 (20.4)	20 (42.6)	18 (46.2)	
BSI (mean ± SD)	5.7 (3.2)	7.7 (3.8)	11.5 (4.0)	<0.001
FACED (mean ± SD)	1.7 (1.2)	1.9 (1.1)	3.73 (1.4)	<0.001

Data are presented as n(%) unless otherwise indicated.

BMI, body mass index; BSI, Bronchiectasis Severity Index; FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity; PPB, potentially pathogenic bacteria; SD, standard deviation.

Table E3.- Sputum LL-37, SLPI, lactoferrin and lysozyme levels in bronchiectasis patients with and without inhaled antibiotic treatment, chronic macrolide therapy and inhaled corticosteroids treatment.

	No chronic macrolides n=104 (77%)	**Chronic macrolides** n= 31 (27%)	**p-value**
Sputum LL-37 (ng/ml)	2440.4 (18.4-7422.1)	26.0 (11.4-10667.6)	0.3
Sputum SLPI (ng/ml)	535.2 (191.6-2610.3)	637.4 (89.9-4042.3)	0.6
Sputum Lactoferrin (µg/ml)	118.5 (50.2-248.3)	55.7 (37.0-300.4)	0.2
Sputum Lysozyme (µg/ml)	69.2 (42.1-106.0)	63.7 (37.2-95.2)	0.7

	No ICS n=76 (56%)	**ICS** n= 59 (44%)	**p-value**
Sputum LL-37 (ng/ml)	1986.3 (17.6-6074.7)	361.9 (12.1-12334.0)	0.6
Sputum SLPI (ng/ml)	659.0 (173.4-4220.4)	509.9 (129.0-2569.0)	0.5
Sputum Lactoferrin (µg/ml)	117.5 (49.5-239.8)	113.5 (40.39-285.0)	0.7
Sputum Lysozyme (µg/ml)	68.4 (37.5-103.0)	75.6 (42.5-106.2)	0.9

	No inhaled antibiotic n= 129 (96%)	**Inhaled antibiotic** n=6 (4%)	**p-value**
Sputum LL-37 (ng/ml)	1443.7 (17.0-8108.1)	2654.7 (29.2-9348.6)	0.3
Sputum SLPI (ng/ml)	560.7 (178.5-3534.5)	246.1 (94.7-861.2)	0.8
Sputum Lactoferrin (µg/ml)	114.6 (46.1-246.0)	136.9 (43.3-385.5)	0.6
Sputum Lysozyme (µg/ml)	69.5 (42.3-105.6)	41.6 (19.1-114.4)	0.5

Data are presented as median (Interquartile range). ICS: Inhaled corticosteroids

Table E4.- Analysis of negative binomial model with adjustments.

		Effect estimate	95% CI	p-value
LL-37 ≥1500 ng/ml		**IRR**		
Exacerbation Rate	Unadjusted	1.57	1.04-2.37	0.02
	Adjusted for BSI	1.53	1.01-2.31	0.04
	Adjusted for site	1.52	1.01-2.28	0.04
	Fully adjusted	1.51	0.97-2.34	0.068
		HR		
Time to first exacerbation	Unadjusted	1.68	1.13-2.50	0.01
	Adjusted for BSI	1.58	1.05-2.36	0.02
	Adjusted for site	1.68	1.11-2.56	0.01
	Fully adjusted	1.72	1.12-2.64	0.01
SLPI <1000 ng/ml		**IRR**		
Exacerbation Rate	Unadjusted	0.76	0.50-1.15	0.1
	Adjusted for BSI	0.77	0.50- 1.17	0.2
	Adjusted for site	0.73	0.48- 1.11	0.1
	Fully adjusted	0.63	0.39-0.98	0.04
		HR		
Time to first exacerbation	Unadjusted	0.63	0.42-0.94	0.02
	Adjusted for BSI	0.68	0.45-1.03	0.06
	Adjusted for site	0.70	0.46-1.05	0.08
	Fully adjusted	0.65	0.41-1.02	0.059

Fully adjusted includes adjustment for bronchiectasis severity, gender, aetiology, inhaled corticosteroid use and site.

IRR= incidence rate ratio, HR= hazard ratio, BSI=Bronchiectasis Severity Index

FIGURE LEGENDS

Figure E1. A: Sputum LL-37 levels of FACED score, divided as mild (0-2 points), moderate (2-4 points) and severe (5-7 points), and B: Sputum SLPI levels of FACED score. p values are obtained by Kruskal-Wallis test. Graphs are represented as mean with SEM.

Figure E2. Relationship between sputum AMP levels (A: LL-37, B: lysozyme, C: lactoferrin and D: SLPI) and FEV_1% predicted. Rho and p-value were obtained from Spearman rank correlation.

Figure E3. A: Sputum LL-37 and B: SLPI levels and frequency of exacerbations in the previous year. p values are obtained by Kruskal-Wallis test. Graphs are represented as mean with SEM.

Figure E4. Relationship between sputum AMP levels (A: LL-37, B: lysozyme, C: lactoferrin) and neutrophil elastase activity (Ns=73). Rho and p-value were obtained from Spearman rank correlation.

Figure E5. A: ROC curve of sputum LL-37 for predicting severe exacerbation (Area under the curve (AUC) 0.76, 95% confidence interval (CI) 0.65-0.86, p<0.0001). B: ROC curve of sputum SLPI levels for predicting severe exacerbation (AUC 0.72, 95% CI 0.62-0.86, p<0.0001).

Figure E1:

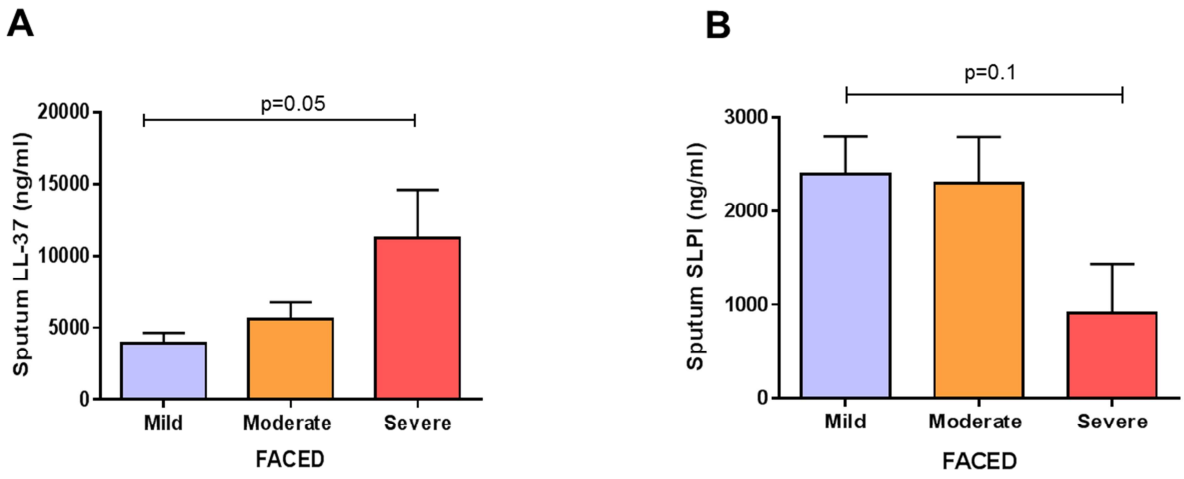

Figure E2:

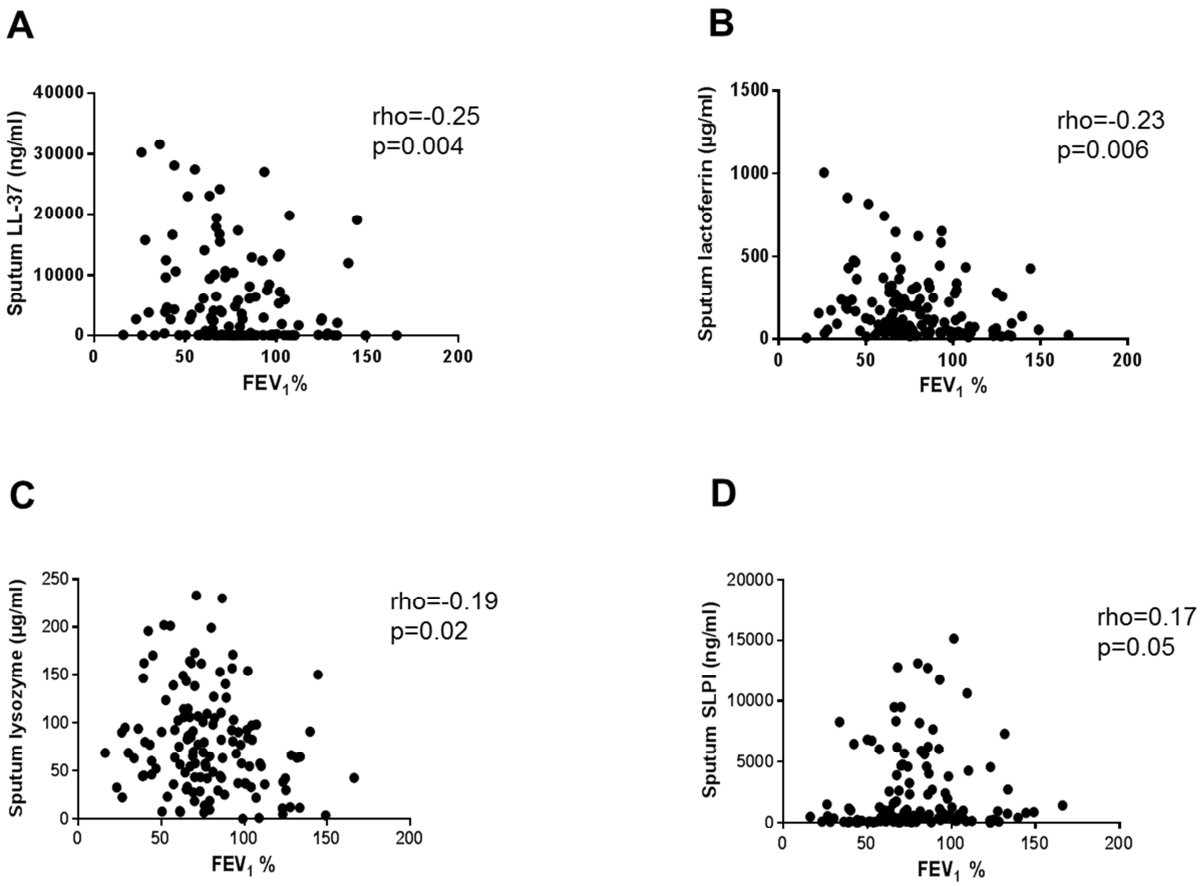

Figure E3:

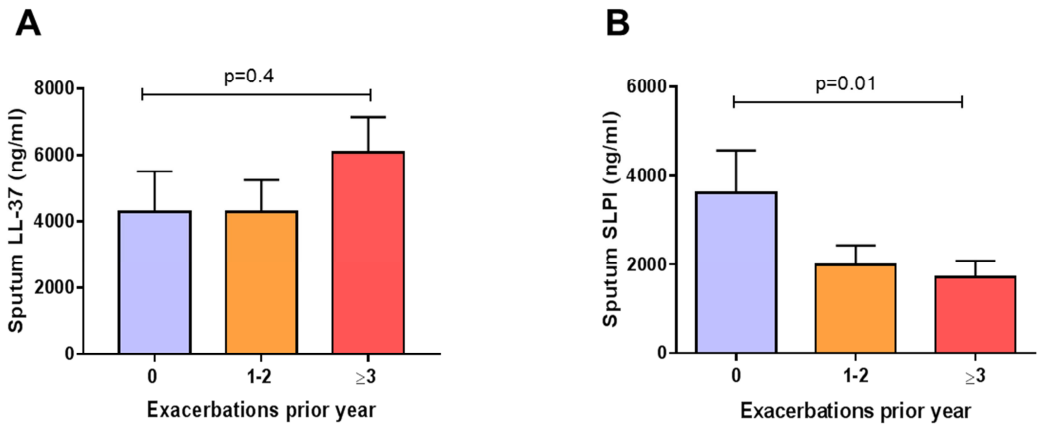

Figure E4:

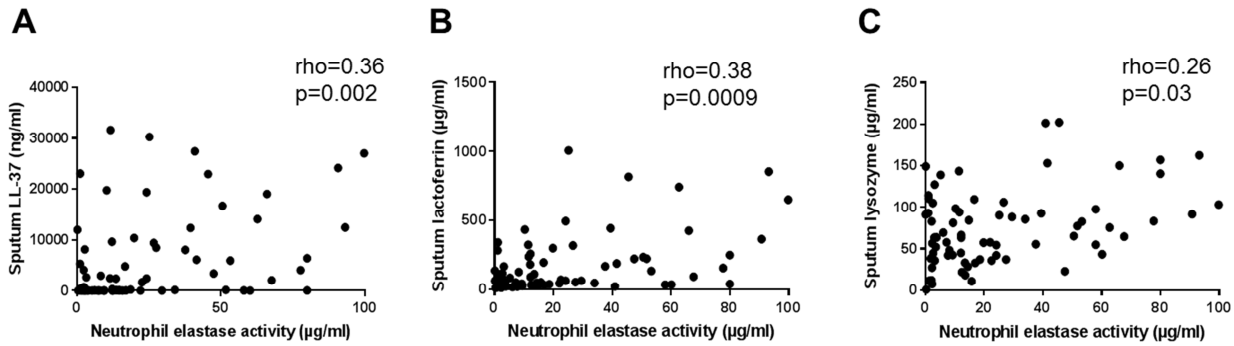

Figure E5: